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AGEING INFLUENCES THE EFFECT OF PRE-HYPOXIC ADMINISTRATION OF CLONIDINE, AN ALPHA$_2$-ADRENOCEPTOR AGONIST, ON POST-HYPOXIC VASOMOTRICITY

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In a previous study, we showed that clonidine, an α_2-adrenoceptor agonist, administered prior to hypoxia improves post-hypoxic contractility (PC) and endothelium-dependent dilatation (PED) in isolated young rat aortas. These effects were not investigated in old rats. Ageing influences vascular physiology and modifies the response to vasoactive drugs. Some drugs, such as simvastatin, improve endothelial function, a pivotal component of vascular homeostasis. This study intends to investigate the effect of pre-hypoxic clonidine administration on post-hypoxic vasomotricity in old rats with or without simvastatin. Isolated aortic rings from young and old rats were submitted to hypoxia/reoxygenation (20 min/40 min). For each aorta ring from one rat, clonidine (10^{-5} M) was administered in two randomised baths and washed out before hypoxia; two other baths constituted the control group. In some experiments, the old rats were treated with simvastatin (10 $mg.kg^{-1}.day^{-1}$) 3 days prior to hypoxia. PED and PC were assessed in all baths. Clonidine enhances PED in young rats ($p<0.001$) but decreases it in old rats ($p=0.038$). In young rats, clonidine improves PC ($p<0.001$), but this effect is not present in old rats ($p=0.339$). Without endothelium, clonidine does not influence PC in young rats ($p= 0.687$) but decreases it in old rats ($p<0.001$). In the simvastatin group, clonidine improves PED ($p<0.001$) but does not influence PC ($p=0.203$). In young rats, clonidine increases PED and PC, while it decreases PED and does not influence PC in old rats. With simvastatin, clonidine improves PED but does not influence PC.

Key words: *ageing, α_2-adrenoceptor agonist, α_2-adrenoceptor, clonidine, hypoxia-reoxygenation, simvastatin, vascular*

INTRODUCTION

Physicians are confronted daily with the ischemia-reperfusion phenomenon (I/R). Vascular and cardiac surgeries, tourniquet use in orthopaedic surgery, thrombolytic therapy for stroke and myocardial infarction, organ transplantation and multiple organ dysfunction syndrome are some examples. The reperfusion of an ischemic organ is essential for its viability, but it induces an endothelial dysfunction that jeopardises its post-ischemic recovery (1-3). The improvement in endothelial function during early reperfusion is an interesting target for protective strategies against I/R injuries. A previous study performed with isolated young rat aorta showed that pre-hypoxic administration of clonidine, an α_2-adrenoceptor agonist, improves both post-hypoxic endothelium-dependent dilatation and contractility, demonstrating the local protective effect on vasomotor tone (4). These findings may partially explain the beneficial effects of α_2-adrenoceptor agonism on the post-hypoxic organ's recovery observed *in vitro* and *in vivo* (5-9). However, aging is associated with many vascular phenotypical modifications in vascular smooth muscle and the endothelium (increased endothelial dysfunction), which affect blood vessel physiology and the vascular response to vasoactive drugs, such as α- and β-adrenergic agonists (10-13). Aged endothelium and vascular smooth cells have increased basal oxidative stress and decreased antioxidant cellular defence capacities, increasing susceptibility to ischemia-reperfusion injuries (14, 15). Some drugs improve endothelial function and decrease hypoxia-reoxygenation. Among these, statins, or 3-hydroxy-3 methyl-glutaryl-CoA reductase inhibitors, have been demonstrated to be beneficial for endothelial function in the elderly animals and humans and to reduce ischemia-reperfusion injury (16-19).

This study investigated the impact of aging on the clonidine-induced improvement in the post-hypoxic vasomotricity observed in young rats. We also investigated the effect of pretreatment with simvastatin on the clonidine effect on post-hypoxic vasomotricity in old rats.

MATERIAL AND METHODS

Animals and aortic ring preparation

All experiments were conducted according to the European Community Guidelines for the experimental animals. Normal chow and tap water were given *ad libitum*. After local animal ethics committee approval (Pr J- Dehoux, Universite Catholique de Louvain, UCL MD 2007-018), young (n=24, 250-300 grams, 4-5 weeks old) and old (n=36, 600-700 grams, 63-64 months old) male Wistar rats were included in the study.

The methodology used for the aortic ring preparation and hypoxic challenge was described in other studies (20). After intra-peritoneal injection of thiopental sodium (50 $mg.kg^{-1}$, Pentothal®, Hospira Enterprises, Hoofddorp, Nederland) and s.c. morphine (10 $mg.kg^{-1}$, Sterop®, Brussels, Belgium), the descending thoracic aorta was quickly removed and placed in warmed Krebs-Henseleit buffer (37°C, composition (in mM): NaCl 118, $NaHCO_3$ 25, KCl 4.8, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, and glucose 11). Adherent tissues were carefully removed, and the aorta was cut into four 3-mm rings. The rings were mounted onto two stainless steel supports suspended in a bath filled with Krebs-Henseleit buffer that was changed every 15 min and bubbled with 95% O_2/5% CO_2 to maintain a PiO_2 between 650 and 700 mmHg and a pH of 7.4. The baths were maintained at 37°C. The rings were attached to a micrometer connected to an isometric force transducer (Power Laboratory 400®, AD Instruments, Casterhill, NSW, Australia). The transducer was linked to an amplifier and a computerised acquisition system (Acknowledge® Software, MP100WSW; Biopac System, Inc., Santa Barbara, CA, USA) to record changes in isometric force. Each aortic ring was equilibrated for 60 minutes with an initial resting tension of 2 grams (young rats) or 2.5 grams (old rats), corresponding to an optimal preload for our rat population. After equilibration, the reproducibility of the contraction for each aortic ring included in the study was tested by several cycles of contraction induced by phenylephrine (10^{-4} M), 4 rinses for 5 minutes each with Krebs-Henseleit buffer, and return to the initial resting tension. When the first contraction reached a plateau, the endothelial integrity was verified by administering acetylcholine (10^{-6}M). In some experiments, the endothelium was removed by gently rubbing the luminal surface of the vessels with a piece of stainless steel wire. The endothelium removal was confirmed by the lack of relaxation in response to acetylcholine in the contracted aorta.

The methodology used was the same for all groups. Each experiment was performed on four aortic segments from the same rat: two were randomised to receive clonidine (10^{-5} M), and the other two constituted the control. After fifteen min. incubation, all of the rings were washed three times (interval 5 min) with warmed Krebs-Henseleit buffer, and the hypoxic challenge was started.

Hypoxic challenge

In all groups, hypoxia-reoxygenation (H/R) was performed. Hypoxia (20 min) was induced by changing the gas mixture to 95% N_2/5% CO_2 (PiO_2 in the bath: 36-38 mmHg after 3 min and remaining stable, pH 7.4). Reoxygenation was performed for 45 min. During this period, each ring was stabilised at its respective resting tension. At the end of reoxygenation, post-hypoxic vasomotricity was tested.

Study groups

Different experiments were conducted to identify the effect of clonidine on young and old rats. The effect of a short pre-treatment with simvastatin on the effect of clonidine on post-hypoxic vasomotricity in old rats was also investigated.

1. Young rats:

Experiment I: Clonidine (10^{-5} M) was added to two randomised baths.

Experiment II: The conditions were identical to experiment I, but the endothelium was removed before the experiment.

2. Old rats:

Experiment III: Clonidine (10^{-5} M) was added to two randomised baths.

Experiment IV: The conditions were identical to experiment III, but the endothelium was removed before the experiment.

Experiment V: All of the rats were pre-treated with oral simvastatin given by gavage at a dose of 10 $mg.kg^{-1}$ once daily and 48 h, 24 h, and 1 h before the experiment. The doses of simvastatin used in previous studies investigating ischemia-reperfusion injuries in rats were very variable (20 $mg.kg^{-1}$-0.5 $mg.kg^{-1}$). For this study, a dose used in a recent study was chosen (21). Clonidine (10^{-5} M) was added to two randomised baths.

Analysis of post-hypoxic vasomotricity.

Post-hypoxic, endothelium-dependent dilatation was investigated on pre-contracted aortic rings (phenylephrine 10^{-4} M) in experiments I, III, and V. After a stable plateau was reached, the aortic rings were exposed to increasing concentrations of acetylcholine (10^{-10}-10^{-4} M) or sodium nitroprusside (10^{-10}-10^{-4} M). Relaxation was expressed as the percentage of the post-hypoxic maximal contraction induced by phenylephrine. Post-hypoxic blood vessel contractility was assessed in all experiments. Aortic rings were exposed to increasing concentrations of phenylephrine (10^{-10}-10^{-4} M). Phenylephrine-induced contraction is expressed as a percentage of the pre-hypoxic maximal contraction in response to phenylephrine (10^{-4} M).

Drugs

All concentrations of the drugs used are expressed as the final molar concentration in Krebs-Henseleit solution. Simvastatin was purchased from Sigma-Aldrich (Bornem, Belgium). Clonidine, acetylcholine, and phenylephrine were obtained from Tocris Bioscience® (Avonmouth, Bristol, United Kingdom).

Statistical analysis

The percentage of maximal contraction or dilation is expressed as the mean ± standard deviation. The effect of clonidine in each of the ten experiments was analysed by regression with generalised estimating equations (GEEs). This method allows for the simultaneous study of the influence of two independent variables: the presence or absence of clonidine and the varying concentration of acetylcholine or phenylephrine according to time (covariate) on the measure of contraction or dilatation in the aortic rings (dependent variable). Unlike classical linear regression, GEE regression is valid when the observations are not independent by taking into account the multiplicity of inter-correlated measurements in each aortic ring.

RESULTS

The effect of pre-hypoxic clonidine administration on post-hypoxic aortic vasomotricity

Experiments I and III

In young rats, clonidine improved post-hypoxic endothelium-dependent dilatation compared to the control group (–33.0±8.3% and –23.8±8.4%, respectively, p=<0.001) (*Fig. 1a*). In contrast, clonidine decreased this measure in old rats (–31.8±3.2% and –35.6±5.8%, respectively, p=0.038) (*Fig. 2a*). Compared to the control group, clonidine did not influence endothelium-independent dilatation in young rats (–58.3±12.3% and –59.0±7.2%, respectively, p=0.703) or in old rats (–58.1±6.3% and –58.4±4.9%, respectively, p=0.454). Clonidine improved post-hypoxic contractility in young rats compared to the control group (65.7±25.5% and 41.6±17.6%, respectively, p<0.001) (*Fig. 1b*). This difference was not observed in old rats (87.2±24.1% and 81.3±12.6%, respectively, p=0.339) *(Fig. 2b*).

Endothelium removal affects clonidine-dependent post-hypoxic vasomotricity

Experiments II and IV

Compared to the control group, endothelium removal abolished the clonidine effect on post-hypoxic contractility in young rats (112.4±24.2% and 111.6±27.4%, respectively, p=0.687) (*Fig. 1c*). In old rats, post-hypoxic contractility was significantly different between the control group and the clonidine group (147.5±76.5% and 95.2±44.3%, respectively, p<0.001) (*Fig. 2c*).

The effect of pre-treatment with simvastatin on clonidine-dependent, post-hypoxic vasomotricity in old rats

Experiment V

In the presence of simvastatin, clonidine improved post-hypoxic, endothelium-dependent dilatation compared to the control group (–31.3±4.4% and –25.0±3.8%, respectively, p<0.001) (*Fig. 3a*). No difference was found in post-hypoxic contractility (69.8±17.3% and 69.4±11.4%, p=0.203) (*Fig. 3b*).

DISCUSSION

Our findings show that clonidine improves post-hypoxic endothelial function and post-hypoxic contractility in young rats. We show also that clonidine increases post-hypoxic endothelial dysfunction and does not influence post-hypoxic contractility in old rats, suggesting that age influences the effect of clonidine on post-hypoxic vasomotricity in isolated old rat aortas. In old rats, pre-treatment with simvastatin restores the beneficial effect of clonidine on post-hypoxic endothelial function but does not influence post-hypoxic contractility.

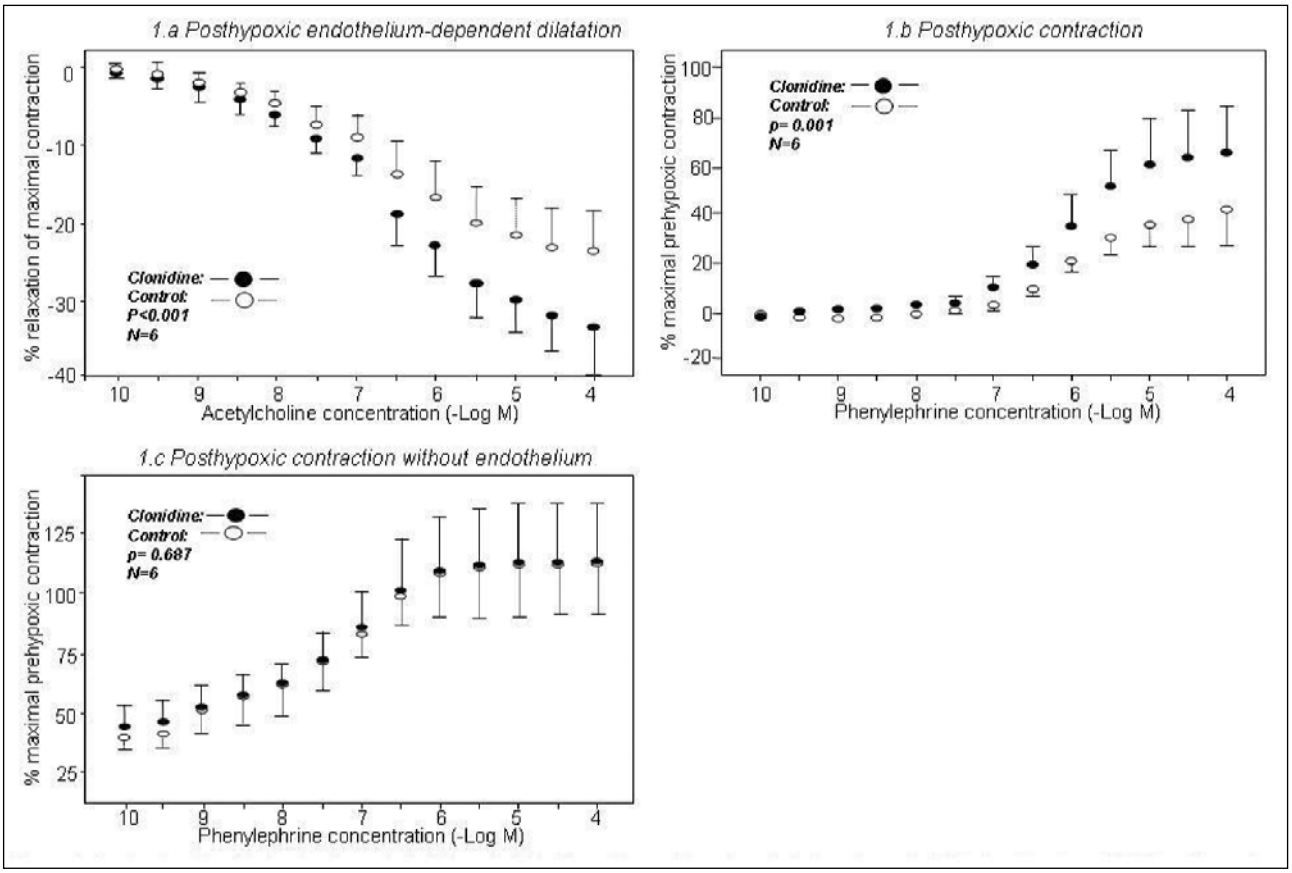

Fig. 1. The graphs show the effects of pre-hypoxic clonidine administration on post-hypoxic endothelium-dependent dilatation (*Fig. 1a*, p<0.001), post-hypoxic contraction (*Fig. 1b*, p<0.001), and post-hypoxic contractility without the endothelium (*Fig. 1c*, p=0.687) in young rats. The clonidine (filled circles) and control (open circles) groups (n=6 each) are presented. The results are presented as the mean ± standard deviation.

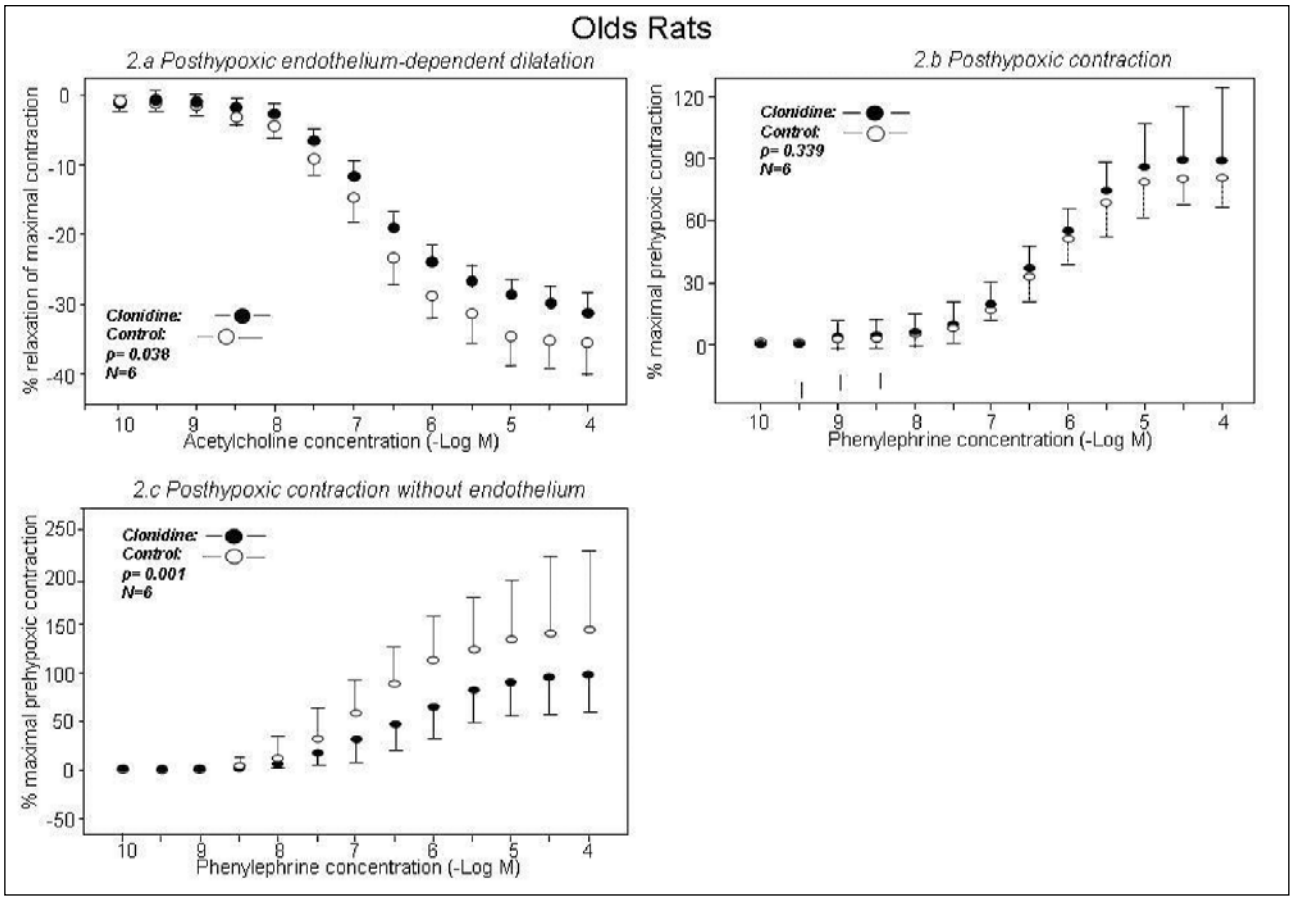

Fig. 2. The graphs show the effect of pre-hypoxic clonidine administration on post-hypoxic endothelium-dependent dilatation (*Fig. 2a*, p=0.038), post-hypoxic contraction (*Fig. 2b*, p=0.339), and post-hypoxic contractility without the endothelium (*Fig. 2c*, p<0.001) in old rats. The clonidine (filled circles) and control (open circles) groups (n=6) are shown. The results are presented as the mean ± standard deviation.

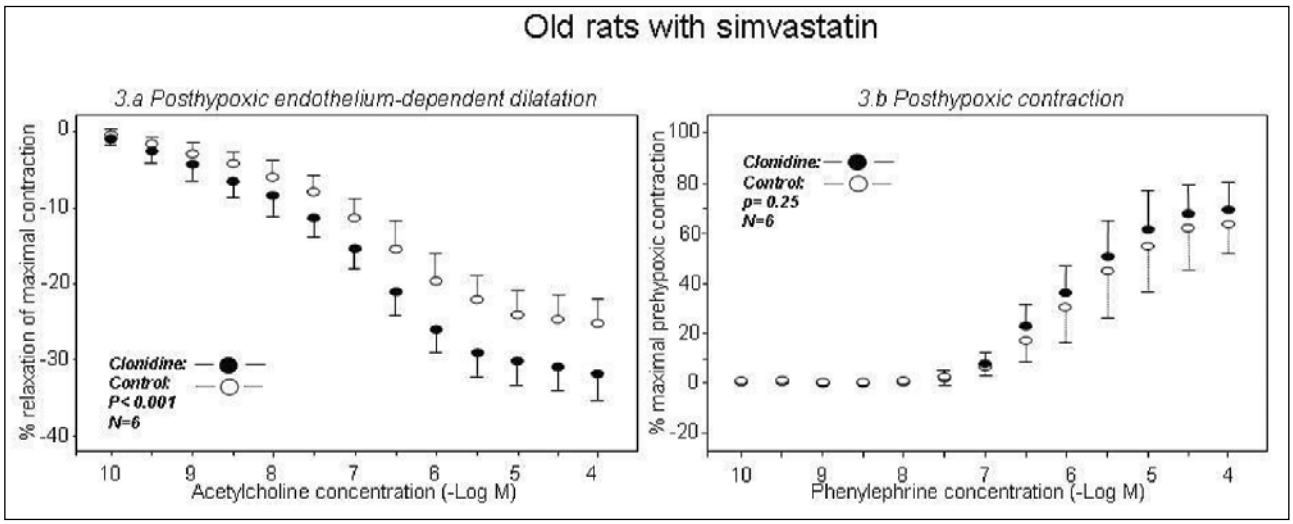

Fig. 3. The graphs show the effect of pre-hypoxic clonidine administration on post-hypoxic endothelium-dependent dilatation (*Fig. 3a*, p<0.001) and post-hypoxic contraction in old rats pre-treated with simvastatin (10 $mg.kg^{-1}.day^{-1}$) (*Fig. 3b*, p=0.203) in the clonidine (filled circles) and control (open circles) groups (n=6). The results are presented as the mean ± standard deviation.

Effect of pre-hypoxic clonidine administration on post-hypoxic, endothelium-dependent dilatation

The results obtained in young rats agree with our previous data (4). The clonidine effect on improving post-hypoxic vasomotricity in young rats involves nitric oxide (NO) synthase and cyclooxygenase (COX) pathways (4). These results are consistent with other studies that have demonstrated that these two intracellular pathways are involved in preconditioning, which is a prosurvival mechanism, and limit ischaemia-reperfusion injury (22-26). NO stimulates soluble guanylate cyclase, which leads to the production of cyclic guanosine

monophosphate and the activation of protein kinase G, directly activating mitochondrial K^+_{ATP} channels, which are putative end-effectors of preconditioning (24, 25). Cyclooxygenases, especially COX-2, are implicated in the early phase of preconditioning in the synthesis of prostaglandin E_2 and/or prostaglandin I_2 (22, 23, 26).

Contrary to observations obtained in young rats, our results show that clonidine increases post-hypoxic endothelial dysfunction in old rats, demonstrating that age influences the effect of clonidine. Ageing alters intercellular communication and transduction pathways, which causes changes in responses to drugs (27). A pivotal endothelial characteristic of senescence is a more pronounced activation of inducible NOS (iNOS), an impaired antioxidant activity and an increased oxidative stress, which reduces nitric oxide bioavailability and causes endothelial dysfunction in the basal state (28, 29). The activity of iNOS progressively increases with age. There is no evidence concerning endothelial NOS (eNOS) activity. Some studies have shown increased expression and activity, while others have described a decrease. However, there is a greater consensus on the decreased eNOS enzyme activity with aging, implicating decreased NO bioavaibility (14). The COXs-dependent pathways are also affected by senescence. During aging, endothelial dysfunction is due in part to the release of endothelium-derived contracting factors that counteract the vasodilator effect of NO. Age-associated oxidative stress enhances the production of eicosanoids and shifts their production/effects from vasodilatation and anti-thrombosis (prostacyclin-PGI_2) to vasoconstriction (thromboxane A_2), prothrombosis and inflammation (30, 31). The activation of COXs by clonidine in old rats could increase the production of vasoconstrictive eicosanoids and be responsible for most of the endothelial dysfunction observed during reoxygenation.

Surprisingly, our findings showed that in ageing rats, simvastatin restores the beneficial effect of clonidine on the post-hypoxic endothelial function observed in young rats. Statins are known to decrease ischemia-reperfusion injury (19, 23, 32, 33). These protective effects seem to be mediated by the inhibition of the mevalonate pathway and modulation of cytokine production (32, 33). Here, we show that simvastatin modifies the effect of a drug in restoring the protective effect of clonidine on endothelial function. Shortly after its administration, simvastatin upregulates different isoforms of the NO synthase enzyme (eNOS, iNOS and neuronalNOS), increasing NO bioavaibility (34). Clonidine can activate eNOS to induce a protective effect on post-hypoxic endothelial function, as seen in young rats.

Effect of pre-hypoxic clonidine administration on post-hypoxic blood vessel contractility

We also investigated the effect of pre-hypoxic clonidine administration on post-hypoxic aortic contractility. Our findings show that the pre-hypoxic administration of clonidine increases aortic post-hypoxic contractility in young rats but does not influence it in old rats. The results obtained in young rats agree with our previous study in which we demonstrated that an intact endothelium and COX are essential to generating the effect of clonidine on post-hypoxic contractility (4). In old rats, endothelial dysfunction, alterations in transduction pathways, and communications between endothelial cells and smooth muscle cells related to age abolished the effect of clonidine on post-hypoxic contractility observed in young rats (27).

Contrary to the results in young rats, endothelium removal dramatically increased post-hypoxic contractility in the control group for the old rats. Clonidine administration prior to hypoxia modulates contractility. These observations suggest a direct action of clonidine on vascular smooth muscle cells. Some studies have shown that α_2-adrenoceptor stimulation in vascular smooth muscle cells hyperpolarises the cell (ATP-dependent potassium channel activation) and limits hypoxia/reoxygenation-induced intracellular calcium overload (35, 36). Moreover, clonidine is also an α_2-adrenergic receptor agonist. Stimulation of this receptor, which is expressed on vascular smooth muscle cells, also limits H/R injury (37). However, these actions seem discrete and are only obvious in the absence of endothelium.

Simvastatin directly acts on vascular smooth muscle cells and limits the production of vasoconstrictive mediators (19, 38). Its anti-oxidant, antiproliferative and calcium mobilisation effects have been reported to be due to the inhibition of isoprenoid intermediate synthesis (39). However, despite its beneficial properties, our results fail to show a clonidine-induced improvement in post-hypoxic contractility in old rats pre-treated with simvastatin.

Our study is descriptive and did not investigate the intracellular mechanism underlying the observed effects. It has several limitations. First, the aorta is a conduit vessel. Although it is sensitive to H/R injuries, its contribution to vascular resistance and blood distribution is minimal. However, no other studies have evaluated the effect of clonidine on the preservation of endothelial function and vasomotricity in old aortas. Second, α_2-adrenergic receptors are widely distributed in the vascular system. This spread varies with species, ageing, vessel diameter, and vascular bed. Considering the heterogeneity in the distribution of α_2-adrenergic receptors, the effect of clonidine might differ in vessels of differing diameter and resistance levels. Further studies are necessary to clarify this issue. Third, these blood vessel studies provide useful but isolated mechanistic information. In the intact animal, clonidine interacts with the sympathetic nervous system, which decreases sympathetic output and catecholamine release through central mechanisms.

In summary, our study demonstrates that clonidine administration before hypoxia improves post-hypoxic, endothelium-dependent dilatation and increases post-hypoxic contractility of the aorta in young rats. However, clonidine administration decreases post-hypoxic, endothelium-dependent dilatation and does not influence post-hypoxic contractility in old rats. When old rats were pre-treated with simvastatin, clonidine improved post-hypoxic, endothelium-dependent dilatation but did not have an effect on post-hypoxic contractility.

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Conflict of interests: None declared.

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